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Molecular Systematics of Malesian *Litsea* Lam. and Putative Related Genera (Lauraceae)

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Phylogenetic relationships within *Litsea* (Lauraceae) and possibly related genera (*Actinodaphne*, *Lindera* and *Neolitsea*) in the tribe Laureae in the Malesian region were investigated at the molecular level. The nucleotide sequences of chloroplast *matK* and the nuclear ribosomal DNA ITS regions were analyzed. The results were compared with sequence data in a publication based on materials from China. Among the four sections recognized in *Litsea*, the molecular data confirmed monophyly only of sect. *Litsea*. Several species of sect. *Conodaphne* were nested within sect. *Cylicodaphne*. Sect. *Tomingodaphne* and sect. *Litsea* were found to be more closely related to several species of the *Lindera* than to *Litsea* sections *Conodaphne* and *Cylicodaphne*. *Actinodaphne* and *Neolitsea*, were found to be monophyletic. Combined analysis of the *matK* sequences of the Malesian and Chinese taxa suggested that a careful re-examination of the taxa in China is necessary. Mapping morphological characters on the molecular trees revealed that dimerous flowers were derived from trimerous flowers and two-celled anthers were derived from four-celled anthers.

Key words: Character evolution, ITS, *Litsea*, Malesia, *matK*, molecular phylogeny

Litsea, with about 400 species, is one of the four largest genera among the 52 genera recognized in the Lauraceae. The other three largest genera are *Cinnamomum*, *Cryptocarya* and *Ocotea*. Each consists of about 350 species (Rohwer 1993). *Litsea* is distinguished from other genera in the same family by having umbellate inflorescences, unisexual and trimerous flowers, usually nine stamens, four-celled anthers, tepals equal to each other and some of them reduced in numbers, fruits with flat to deeply cup-shaped cupules, and leaves alternate or sometimes opposite (van der Werff 2001). Species of *Litsea* occur mostly in Asia, with several species in Australia and the Pacific islands, and a few in America (Rohwer 1993).

Taxonomists have different opinions on the infrageneric relationship of *Litsea*. According to Bentham (1880), *Litsea* can be divided into four sections: Sect. *Eulitsea* Benth. defined as having incomplete or absent perianth segments, a perianth tube not enlarged or only slightly enlarged in fruits, and often more than 12 stamens; Sect. *Conodaphne* (Blume) Benth. with complete perianth segments, usually nine stamens and a flat to slightly enlarged perianth tube in fruits; Sect. *Neolitsea* Benth. with triplinerved leaves, dimerous flowers, four perianth segments, six stamens and fruits similar to those of sect. *Conodaphne*; Sect. *Cylicodaphne* (Nees) Benth. with penninerved leaves, trimerous flowers, six perianth segments, 12 stamens and an enlarged perianth tube with cup-shaped cupules. Hooker (1890) used deciduous versus persistent leaves to distinguish a fifth section, *Tomingodaphne* (Blume) Hook. f.

Based on the same characters as in previous classifications, Pax (1891) transferred sect. *Neolitsea* from *Litsea* to a new genus, *Tetradenia* Pax. He retained the other four sections recognized by Hooker (1890) under *Litsea* in his classification. Kostermans (1957) divided *Litsea* into three subgenera: plants of subgen. *Litsea* are monoecious; those of subgen *Dodecadenia* (Nees) Kosterm. are bisexual; and those of subgen. *Octolitsea* Liou-Ho are dioecious. The flowers in subgen. *Octolitsea* have eight tepals. Kostermans recognized *Neolitsea* as distinct from *Litsea*.

Based on Chinese species, Li *et al.* (1982) divided *Litsea* into two subgenera: *Litsea* and *Uniflos* Yang et P. H. Huang. Within subgen. *Litsea* they recognized four sections: *Litsea*, *Tomingodaphne* (Blume) Hook. f., *Conodaphne* (Blume) Benth. and *Cylicodaphne* (Nees) Benth. The subgenus *Uniflos* is monotypic, represented by *Litsea monantha* Yang et P. H. Huang. They also considered *Neolitsea* to be distinct. Subsequently, Li (1985) recognized *L. monantha* of subgen. *Uniflos* of Li *et al.* (1982) to be conspecific with *Dodecadenia grandiflora* Nees, leaving *Litsea* with the four sections of Li *et al.* (1982) minus subgen. *Uniflos*.

A close relationship between *Litsea*, *Actinodaphne*, *Lindera* and *Neolitsea*, has been suggested by Li (1995), Rohwer (2000) and Chanderbali *et al.* (2001). The study by Li (1995) was based on morphological analysis, while the other two studies were based on molecular analysis. Li (1995) used the number of anther cells (2 vs. 4) and flower arrangement (dimerous vs. trimerous) to infer the phylogenetic relationship among these genera. The number of anther cells has been one of the most important characters in the classification of Lauraceae (e.g. Bentham 1880, Kostermans 1957, Huchinson 1964). Instability of this character was recently found in some genera (van der Werff & Richter 1996), making relationships and delimitations of *Litsea* and related genera obscure.

A comprehensive systematic study of *Litsea* by Li & Christophel (2000) using gross morphological and leaf cuticle characters attempted to elucidate the relationships of *Litsea* and related genera. This group, referred to as the *Litsea* complex, consists of 10 genera: *Litsea*, *Lindera*, *Neolitsea*, *Actinodaphne*, *Dodecadenia*, *Iteadaphne*, *Parasassafras*, *Sinosassafras*, *Umbellularia* and *Laurus*. Most of these genera are members of the tribe Laureae of van der Werff and Richer (1996). The outcome of the study by Li & Christophel (2000) did not clearly show the relationships within the complex. Based on the molecular trees obtained from nucleotide sequences of the *matK* gene of the chloroplast genome and the internal transcribed spacer (ITS) regions of ribosomal DNA of the nuclear genome for the same species examined by Li & Christophel (2000), Li *et al.* (2004) concluded that most genera of the *Litsea* complex are polyphyletic. Conflicts between their *matK* and ITS data, however, gave low bootstrap support for their combined tree. Their molecular analysis, however, was based on only four species of *Litsea*.

Despite the above studies, the delimitation, phylogenetic relationships, systematics and evolution of the *Litsea* complex remain poorly understood. Owing to the wide distribution, the large number of species and extensive morphological variation, the relationships of the sections in *Litsea* and related genera are still problematic. To understand the relationships, we decided to focusing on the species of a particular geographic area, the Malesian region, where the Lauraceae are an important component of many forests and where *Litsea* is one of the largest genera. The number of species of *Litsea* is still unknown and no monograph exists for the region. Our study did not aim to resolve the phylogenetic relationships of the entire *Litsea* complex, but instead to understand the infrageneric relationships of *Litsea* in the Malesian region using molecular data to provide a basis for further studies of the genus within the region. For this purpose, we examined materials from Indonesia and Malaysia. Most of the species we examined are widely distributed. We also included some of the related genera mentioned above. Of the 10 genera in the *Litsea* complex (Li & Christophel 2000; Li *et al.* 2004), only five occur within the Malesian region: *Actinodaphne*, *Lindera, Litsea, Neolitsea*, and *Iteada-*

phne. We excluded *Iteadaphne*, since this monotypic genus is now considered more closely related to *Lindera* than to *Litsea* (Kosterman 1957; Tsui 1987; van der Werff 2001). In our study, we sequenced the *matK* and ITS regions for molecular phylogenetic analyses, since these regions have been useful for resolving intergeneric and infrageneric relationships (Soltis & Soltis 1998). Although these regions gave conflict results in Li *et al.* (2004), our preliminary study using a BLAST program search (Altschul *et al.* 1997) revealed that some of the ITS sequences of Li *et al.* (2004) showed significant sequence homology with fungal sequences. We suspect that a fungal contaminant in some of their samples may explain the discrepancies in their results.

The main objectives of our study were (1) to provide a molecular phylogenetic tree of *Litsea* and related genera using materials from part of the Malesian region, (2) to compare our data with those of the previous study by Li *et al.* (2004) using Chinese materials, and (3) to discuss character evolution of a number of morphological traits formally used for classification of this plant group.

Materials and Methods

Plant materials

We performed molecular analysis of 39 species of *Litsea*, *Lindera*, *Neolitsea* and *Actinodaphne*, which are the main genera of the Laureae distributed in the Malesian region. We examined 24 species of *Litsea*, one species of sect. *Tomingodaphne*, two species of sect. *Litsea*, nine species of sect. *Conodaphne* and 12 species of sect. *Cylicodaphne*. We also included six species of *Actinodaphne*, four species of *Neolitsea* and five species of *Lindera* in our study. The plants were collected in the Bogor Botanic Garden and Cibodas Botanic Garden in Indonesia, Lambir National Park in Malaysia, and in Kyoto and Kochi in Japan. We used *Machilus rimosa* and *Phoebe exelsa* (tribe Perseeae) as outgroups, because tribe Perseae has been shown to be a sister to the tribe Laureae, to which our taxa belong (Rohwer 2000, Chanderbali *et al.* 2001). A complete list of the species examined in this study, along with voucher, GenBank/DDBJ/EMBL accessions and source information is in Table 1.

DNA extraction

Leaf samples for DNA extraction were collected in the field or from cultivated plants and dried in silica gel. Total DNA was extracted following the procedure of Kawahara *et al.* (1995). Polysaccharides and oils were removed using washing buffer solution (0.1 M HEPES pH 8.0, 2% 2-mercaptoethanol, 1% polyvinylpyrrolidone and 0.05 M ascorbic acid). Some DNA samples required further purification using a Qiagen-tip 20 column (Qiagen, Hilden, Germany) and we applied the procedures described in Kawahara *et al.* (1995).

Polymerase chain reaction (PCR) amplification and nucleotide sequencing

Double-stranded DNA of the chloroplast *matK* region was amplified using the primer pair of *trnK* 3914 and *trnK* 2R (Johnson & Soltis 1995). The amplification reaction used anti-Taq high polymerase (TOYOBO, Osaka, Japan) in a total volume of 25 μL. The PCR profile consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 3 min, with a final extension at 72°C for 7 min on a Model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

The amplification of the nuclear ITS region of double-stranded DNA was carried out using the primer pair of LAUR1 (Chanderbali *et al.* 2001) and ITSB (Blattner 1999). The amplification reaction used TaKaRa Ex Taq™ DNA polymerase (TaKaRa Bio, Otsu, Shiga, Japan); 10% DMSO was added to reach a total volume of 20 μL. The PCR profile consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min on a Model 9700 thermal cycler.

After amplification, the PCR products were checked by electrophoresis in 1.0% agarose gels

TABLE 1. Plant materials examined in this study. The materials were collected from Bogor Botanical Garden (BBG) and Cibodas Botanical Garden (CBG), Indonesia; Lambir National Park (LNP), Malaysia; Kochi (KOC) and Kyoto (KYO), Japan. The samples were collected by I.A. Fijridiyanto (IZ) or T. Iwasaki (TI). Vouchers are deposited in the Makino Herbarium, Tokyo Metropolitan University (MAK).

Species	Voucher	Source	Origin/Range	Accession No. *matK*/ITS
Actinodaphne glomerata (Blume) Nees	*IZ 802*	BBG	West Malesia	AB258991/AB260849
Actinodaphne macrophylla (Blume) Nees var. *angustifolia* Koord. & Valet.	*IZ 854*	BBG	Peninsular Malaysia to Papua	AB258990/AB260850
Actinodaphne maingayi Hook. f.	*IZ 2068*	LNP	Borneo	AB259062/AB260851
Actinodaphne malaccensis Hook. f.	*IZ 2053*	LNP	Borneo	AB258992/AB260852
Actinodaphne myriantha Merr.	*IZ 2052*	LNP	Borneo	AB259063/AB260853
Actinodaphne procera Nees	*IZ 2057*	LNP	Borneo	AB259064/AB260854
Lindera erythrocarpa Makino	*TI 526*	KOC	Japan	AB259065/AB260855
Lindera lucida (Blume) Boerl.	*IZ 2010*	LNP	Borneo	AB259066/AB260856
Lindera obtusiloba Blume	*TI 3402*	KYO	Japan	AB259067/AB260857
Lindera polyantha (Blume) Boerl.	*IZ 876*	CBG	West Java	AB259068/AB260858
Lindera umbellata Thunb.	*TI 3477*	KYO	Japan	AB259069/AB260859
Litsea accedens (Blume) Boerl.	*IZ 2066*	LNP	Borneo	AB259070/AB260860
Litsea caulocarpa Merr.	*IZ 2043*	LNP	Borneo	AB259071/AB260861
Litsea costalis (Nees) Kosterm.	*IZ 2041*	LNP	Borneo	AB259072/AB260862
Litsea cubeba (Lour.) Pers.	*IZ 863*	CBG	Cochinchina, China	AB259073/AB260863
Litsea diversifolia Blume	*IZ 864*	CBG	West Java	AB259074/AB260864
Litsea erectinervia Kosterm.	*IZ 2032*	LNP	Borneo	AB259075/AB260865
Litsea fenestrata Gamble	*IZ 2031*	LNP	Borneo	AB259076/AB260866
Litsea ferruginea (Blume) Blume	*IZ 2016*	LNP	Borneo	AB259077/AB260867
Litsea firma Hook. f.	*IZ 835*	BBG	West Kalimantan	AB259078/AB260868
Litsea garciae S.Vidal	*IZ 2025*	LNP	Borneo	AB259081/AB260869
Litsea globularia Ng	*IZ 2044*	LNP	Borneo	AB259079/AB260870
Litsea glutinosa (Lour.) C. B. Rob.	*IZ 824*	BBG	Malesia	AB259080/AB260871
Litsea grandis (Wall.) Hook. f.	*IZ 2042*	LNP	Borneo	AB259082/AB260872
Litsea lancifolia Hook. f. var. *grandifolia* (Stapf) Ng	*IZ 2047*	LNP	Borneo	AB259083/AB260873
Litsea machilifolia Gamble	*IZ 2037*	LNP	Borneo	AB259084/AB260874
Litsea maingayi Hook. f.	*IZ 2007*	LNP	Borneo	AB259085/AB260875
Litsea mappacea (Blume) Boerl.	*IZ 871*	CBG	West Java	AB259086/AB260876
Litsea noronhae Blume	*IZ 818*	BBG	Java	AB259087/AB260877
Litsea ochracea (Blume) Boerl.	*IZ 2034*	LNP	Borneo	AB259088/AB260878
Litsea resinosa Blume	*IZ 839*	BBG	Sumatra	AB259089/AB260879
Litsea rubicunda Kosterm.	*IZ 2026*	LNP	Borneo	AB259090/AB260880
Litsea sarawacensis Gamble	*IZ 2049*	LNP	Borneo	AB259091/AB260881
Litsea tomentosa Blume	*IZ 874*	CBG	West Java	AB259092/AB260882
Litsea umbellata Merr.	*IZ 809*	BBG	West Java	AB259093/AB260883
Machilus rimosa Blume	*IZ 870*	CBG	Java	AB259098/AB260888
Neolitsea aciculata (Blume) Koidz.	*IZ 1001*	KYO	Japan	AB259094/AB260884
Neolitsea cassia (L.) Kosterm.	*IZ 831*	BBG	Sumatra	AB259095/AB260885
Neolitsea javanica (Blume) Backer	*IZ 869*	CBG	West Java	AB259096/AB260886
Neolitsea sericea (Blume) Koidz.	*IZ 852*	BBG	Java	AB259097/AB260887
Phoebe excelsa Nees	*IZ 868*	CBG	Java	AB259099/AB260889

and the amplified fragments were purified using a QIAquick Gel Extraction Kit (Qiagen). For nucleotide sequencing, the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used. The cycling consisted of an initial denaturation at 96°C for 10 s, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 s. Sequencing was performed using a 3100 Genetic Analyzer (Applied Biosystems). All primers used for sequencing in this study are listed in Table 3.

Sequence alignment

The nucleotide sequences of the *matK* and ITS regions obtained for each plant species were aligned using Auto Assembler ver. 1.4.0 software (Applied Biosystems). After the overlapping sequences had been checked, a connected sequence for each species was generated. The connected sequences for all taxa were realigned using Sequence Navigator ver. 1.0.1 (Applied Biosystems), and then adjusted manually following guidelines in Kelchner (2000) to produce a final data matrix. We performed a homology search for all species using the BLAST program (Altschul *et al.* 1997) to verify that the samples were free of contamination.

To compare our data with data from different geographical regions, we created a combined data set using data by Li *et al.* (2004), which were mostly obtained from samples from China. Their sequence data were retrieved from the DNA data-

TABLE 3. Primers used for PCR amplification and sequencing of *matK* and ITS regions.

	Primer sequence 5′–3′	Source
matK		
Forward		
909(*trnK*3914F)	GGGGTTGCTAACTCAACGG	Johnson & Soltis (1995)
448	GTGTCAGATATACTAATACC	Rohwer (2000)
805	ACCCTATGGTTGTTCAAAGAC	Rohwer (2000)
1084	CTATTAAGAAATTCGAGACC	Rohwer (2000)
1318	TGTGCTAGAACTTTGTCTCG	Rohwer (2000)
matK-AF	CTATATCCACTTATCTTTCAGGAGT	Ooi *et al.* (1995)
matK-BF	TCAGAGGGATTTGCGTTTATTGTGG	Ooi *et al.* (1995)
Reverse		
2288(*trnK*-2R)	AACTAGTCGGATGGAGTAG	Johnson & Soltis (1995)
805	GTCTTTGAACAACCATAGGGT	Rohwer (2000)
941	CCGGTTGAGACCACAAGT	Rohwer (2000)
1166	ACGGCTTACTAATGGGATGCC	Rohwer (2000)
1422	TTGGGAAGATCAAAGAAAGA	Rohwer (2000)
1847	ACTAGTCGGATGGAGTAGA	Rohwer (2000)
matK-R	CTGCATATACGCCCAAATCGGTCAA	Ooi *et al.* (1995)
matK-8R	AAAGTTCTAGCACAAGAAAGTCGA	Ooi *et al.* (1995)
ITS		
Forward		
LAUR1	ACCACCACCGGCAACCA	Chanderbali *et al.* (2001)
ITS3	GCATCGATGAAGAACGCAGC	White *et al.* (1990)
Reverse		
ITS2	GCTACGTTCTTCATCGATGC	White *et al.* (1990)
ITSB	CTTTTCCTCCGCTTATTGATATG	Blattner (1999)

TABLE 2. DNA sequence of *matK* data analyzed by Li *et al.* (2004) included in the analyses of this study.

Species	Accession No.
Litsea glutinosa (Lour) C. B. Rob.	AF 244396
Litsea umbellata (Lour) Merr.	AF 244395
Litsea dilleniifolia P. Y. Pai & P. H. Huang	AF 244397
Lindera megaphylla Hemsl.	AF 244404
Lindera reflexa Hemsl.	AF 244401
Lindera metcalfiana Allen	AF 244403
Lindera fruticosa Hemsl.	AF 244405
Lindera obtusiloba Blume	AF 244402
Lindera communis Hemsl.	AF 244406
Actinodaphne obovata (Nees) Blume	AF 244410
Actinodaphne forrestii (Allen) Kosterm.	AF 244411
Neolitsea confertifolia (Hemsl.) Merr.	AF 244394
Neolitsea levinei Merr.	AF 244393
Iteadaphne caudata (Nees) H. W. Li	AF 244408
Parasassafras confertiflora (Meissn.) Long	AF 244392
Sinosassafras flavinervia (Allen) H. W. Li	AF 244390
Laurus nobilis L.	AF 244407
Sassafras tzumu (Hemsl.) Hemsl.	AF 244391
Umbellularia californica (Hook. & Arn.) Nutt.	AF 244389

base of DDBJ (http://www.ddbj.nig.ac.jp). The combined data sets were analyzed separately from sets based on only our data. We included 20 of the 23 species of Li *et al.* (2004) in the combined *matK* data set. Sequence data for the other three species were not included in our analyses because there were several "N" characters in their *matK* sequences. A list of the DNA sequence data reported by Li *et al.* (2004), and which were used for comparison in this study, is shown in Table 2.

We combined our data with only the *matK* data of Li *et al.* (2004), since their ITS data needed further clarification, as indicated by our homology search using the BLAST program. The results showed that the sequences from their samples, such as from *Lindera tienchuanensis* (AY265412), *Neolitsea confertifolia* (AY265400) and *Sinosassafras flavinervia* (AY265394), may have contained errors due to contamination by one or more species of *Aspergillus*. Their data also showed ITS sequences from different genera (e.g. *Actinodaphne forrestii* and *Lindera megaphylla*) to be too similar. We did not, therefore, use the ITS data from the studies of Li *et al.* (2004).

Phylogenetic analyses

Phylogenetic analyses based on maximum parsimony criteria were performed using PAUP* version 4.0b10 (Swofford 2002) for two kinds of data sets. The first analysis was based only on our own data sets, which consisted of *matK*, ITS, and a combination of the two. The second analysis was based on combined *matK* data, ours and those of Li *et al.* (2004). Insertions and deletions were treated as missing data. All characters were equally weighted and unordered (Fitch 1971). Both data sets were analyzed by the heuristic search method with tree bisection-reconnection (TBR) branch-swapping and the MULTREES option on, ten replications of sequence addition with the stepwise addition option, and all of the most parsimonious trees (MPTs) were saved. The evaluation of internal support of clades was conducted by bootstrap analysis (Felsenstein 1985)

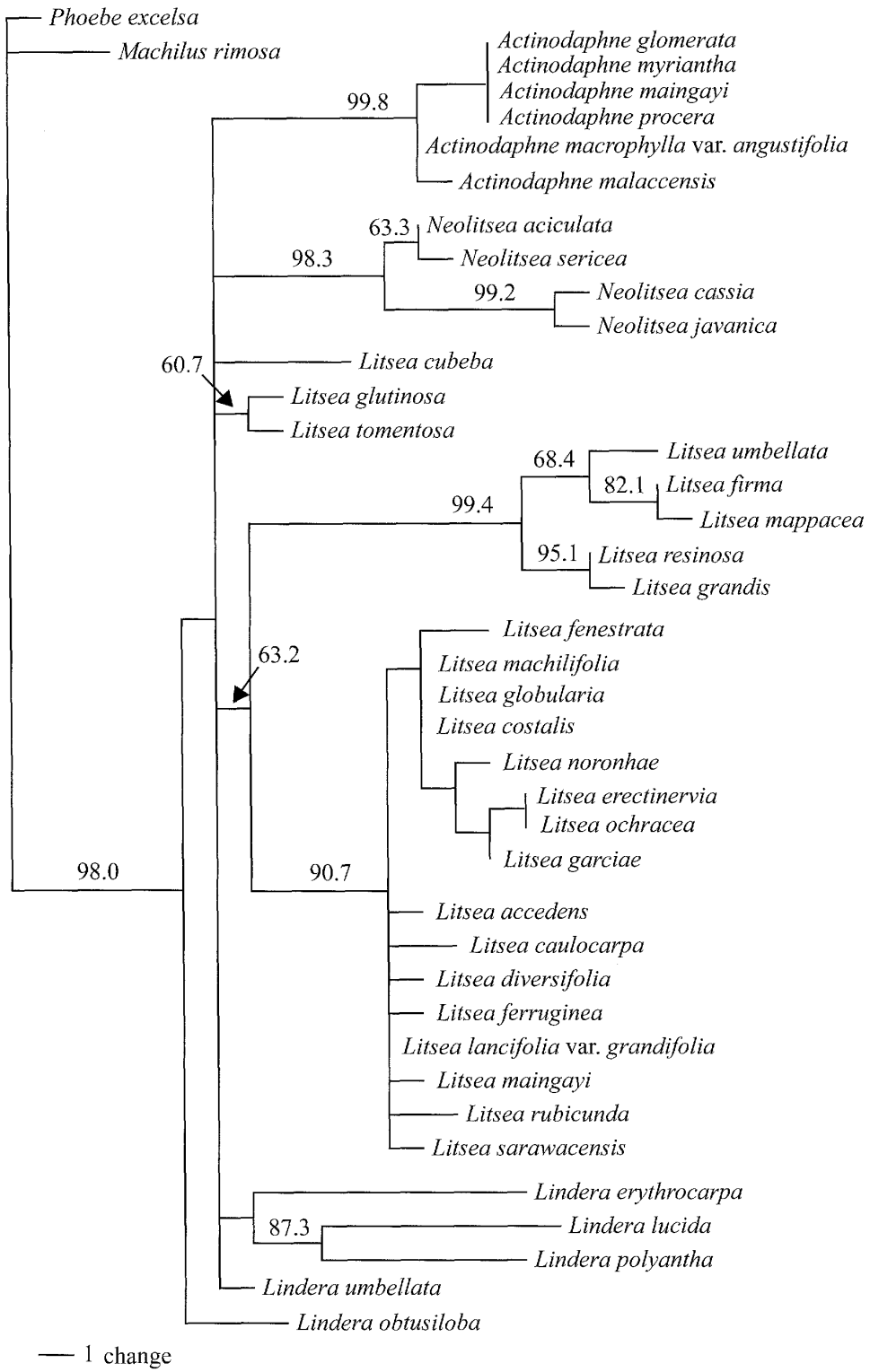

FIG. 1. One of 66 most parsimonious trees derived from analysis of *matK* sequences (length = 107; CI = 0.897; RI = 0.929). Internal support was examined by bootstrap analysis of 1,000 replicates. Bootstrap values are shown above branches. Branch length corresponds to number of nucleotide substitutions; scale bar is shown on lower left.

utilizing 1,000 replicates with TBR branch-swapping and the MULTREES option off. The number of steps, consistency indices (CI) and retention indices (RI) were calculated using one of the MPTs in each analysis using the TREE SCORE command in PAUP*. The congruence between *matK* and ITS data was tested with the incongruence length differences (ILD) test (Mickevich &

Faris 1981, Faris *et al.* 1994) as implemented in PAUP* (the "partition-homogeneity test").

Most parsimonious reconstruction (MPR) of character evolution

Two phenetic characters (arrangement of flower parts and number of anther cells), which have been used to distinguish genera of the *Litsea* complex, were mapped onto the molecular phylogenetic tree obtained from our data matrix of the combined *matK* and ITS sequences using Mesquite ver. 1.06 (Maddison & Maddison 2005). Morphological data were based on information in published floras (Hooker 1890, Ridley 1924, Backer & van den Brink 1963, Ohwi 1965, Li *et al.* 1982) as well as on our own observation of herbarium specimens deposited in the herbaria of Kyoto University (KYO) and the Sarawak Forest

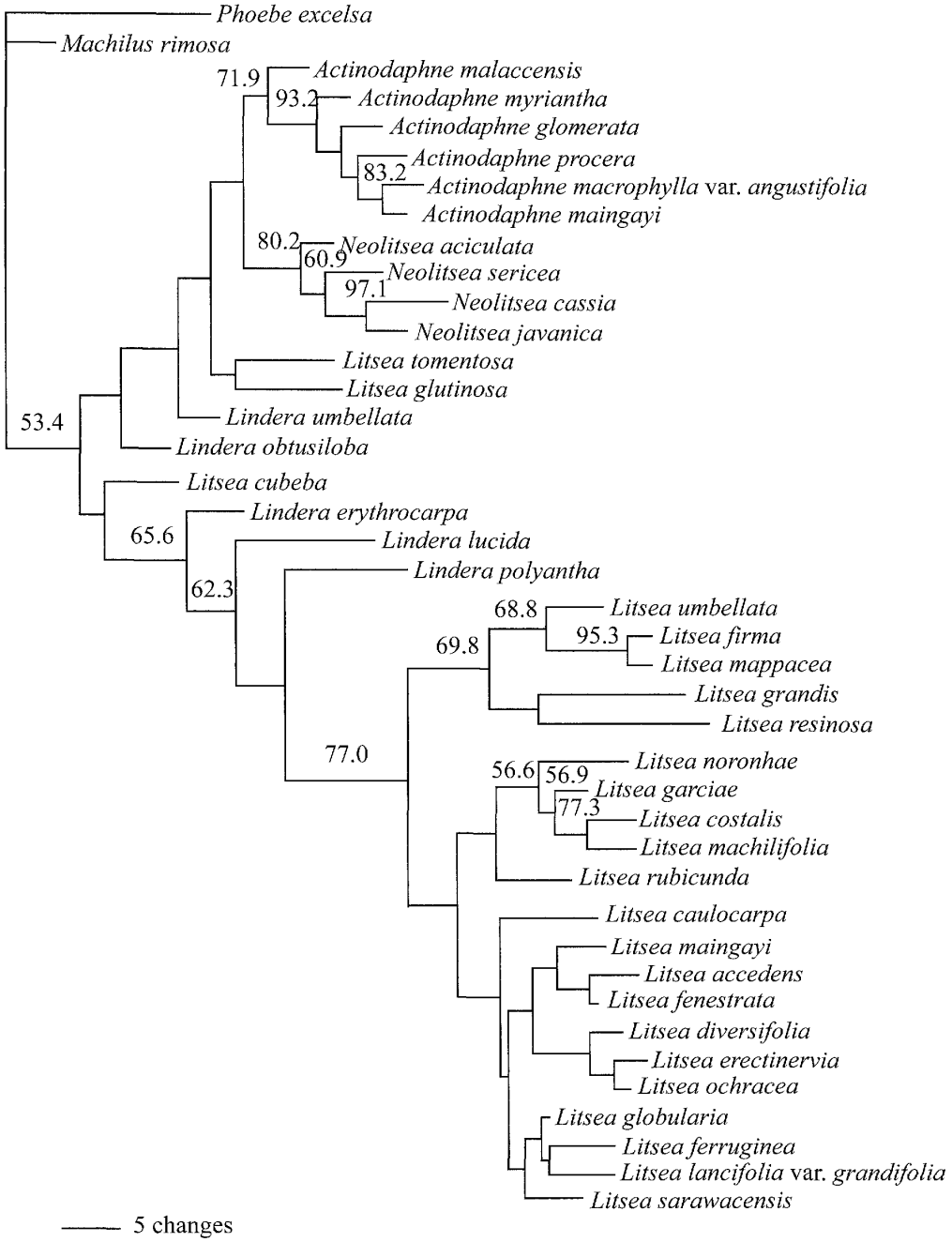

FIG. 2. One of six most parsimonious trees derived from analysis of ITS sequences (length = 506; CI = 0.484; RI = 0.642). Internal support was examined by bootstrap analysis of 1,000 replicates. Bootstrap values are shown above branches. Branch length corresponds to number of nucleotide substitutions; scale bar is shown on lower left.

Research Center (SAR).

Results

Nucleotide sequences of matK *and phylogenetic analysis*

The aligned nucleotide *matK* sequences, including small parts of the *trnK* intron comprised 1,628 characters. Among these, 1,534 (94.2%) were constant, 46 (2.8%) were parsimony-uninformative and 48 (2.9%) were parsimony-informative characters (Table 4). The nucleotide sequence of the four species of *Actinodaphne* was identical. Two pairs of identical sequences were also found in *Litsea*, one of which was shared by three and the other by two different species. The parsimony analysis resulted in 66 MPTs with a length of 107 steps, CI of 0.897 and RI of 0.929. One of the most parsimonious trees with bootstrap percentages (BP) is shown in Fig. 1. Monophyly was shown for *Actinodaphne* with a BP of 99.8%, and for *Neolitsea* with a BP of 98.3%. *Litsea* was divided into three main lineages with BPs of 60.7%, 99.4% and 90.7%. The latter two lineages formed a clade with a low BP support of 63.2%. *Litsea cubeba* did not join with any of these three lineages. In *Lindera,* only two species (*Lindera polyantha* and *Lindera lucida*) were shown to be closely related, with moderate BP support of 87.3%. Relationships among the other species of *Lindera* were not clear based on this analysis.

Nucleotide sequences of ITS and phylogenetic analysis

We removed some variable sites from our ITS sequence data set because of difficulties in aligning them. The resultant ITS data set contained ITS1, 5.8S and ITS2 sites. It comprised 632 characters after alignment. Of these, 447 (70.7%) were constant, 74 (11%) were parsimony-uninformative and 111 (17.6%) were parsimony- informative characters (Table 4). The most parsimonious analysis resulted in six MPTs with a length of 506 steps, CI of 0.484 and RI of 0.642. One of the most parsimonious trees demonstrated that only a few groups were supported by BPs greater than 50% (Fig. 2). The tree in Figure 2 also showed that both *Actinodaphne* and *Neolitsea* were monophyletic with BP values of 71.9% and 80.2%, respectively. In *Litsea*, most species were joined in one clade with BP 77%, except for *Litsea cubeba*, *Litsea tomentosa* and *Litsea glutinosa*, whose positions remained obscure. The relationships among the species of *Lindera* were also unclear.

Combined analysis of matK *and ITS data*

The combined *matK* and ITS data sets passed the ILD test (P = 0.987), suggesting that nuclear and chloroplast data sets are congruent for the data set as a whole (Darlu & Lecointre 2002). Combined analysis can produce results superior to single gene analysis (Gontcharov *et al.* 2004). Our analysis of the combined data sets provided a better resolved tree than any individual data set.

The aligned matrix for the combined *matK*

TABLE 4. Statistics calculated from parsimony analyses of the separate and combined data matrices of *matK* and ITS as well as combined *matK* with those of Li *et al.* (2004).

	matK	ITS	*matK* & ITS	*matK* with Li *et al.* (2004)
No. of sites	1,628	632	2,260	1,405
No. of constant sites (%)	1,534 (94.2%)	447 (70.7%)	1,982 (87.7%)	1,292 (91.9%)
No. of variable sites (%)	46 (2.8%)	74 (11.7%)	116 (5.1%)	68 (4.8%)
No. of informative sites (%)	48 (2.9%)	111 (17.6%)	162 (7.2%)	45 (3.2%)
No. of steps (substitution)	107	506	620	125
No. of MPTs	66	6	12	180
CI	0.897	0.484	0.548	0.912
RI	0.929	0.642	0.699	0.932

and ITS analysis comprised 2,260 characters. The analysis resulted in 12 MPTs with a length of 620 steps, CI of 0.548 and RI of 0.699 (Table 4). The strict consensus tree of MPTs is shown in Fig. 3. According to the tree obtained, the monophyly of both *Actinodaphne* and of *Neolitsea* was also supported by BPs of 98.5% and 99.3%, respectively. These two genera showed a sister group relationship, with a BP of 54.1%. In *Litsea*, members of sect. *Litsea* were in the same clade, with a BP of 58.3%. Section *Tomingodaphne*, represented by only one species (*L. cubeba*), was in the same lineage with some species of *Lindera* but with a BP less than 50%. Members of sect. *Conodaphne* were divided into two clades. Species with alternate leaves of sect. *Conodaphne* formed a clade with a BP of 99.7%, while species with opposite leaves of sect. *Conodaphne* were nested with members of sect. *Cylicodaphne* with a BP of 85.9%. Those two clades were sisters, with a BP of 89.6%. In *Lindera,* three species (*Lindera polyantha*, *Lindera lucida* and *Lindera erythocarpa*) were shown to be closely related to each other with a BP support of 81.2%.

Phylogenetic analysis of matK *data combined with data from Li* et al. *(2004)*

The combined *matK* data, which included data from Li *et al.* (2004), resulted in aligned sequences of 1,405 characters. Of these, 1292 (91.9%) were constant, 68 (4.8%) were parsimony-uninformative and 45 (3.2%) were parsimony-informative characters. The analysis resulted in 180 MPTs with a length of 125 steps, CI of 0.912 and RI of 0.932 (Table 4). One of the most parsimonious trees is shown in Fig. 4. *Neolitsea*, including species analyzed by Li *et al.* (2004), was shown to be a monophyletic group BP support of 85.6%. The polyphyly of many genera of the *Litsea* complex is shown in the tree obtained. For example: *Lindera metcalfiana* was nested in the Malesian *Actinodaphne* group with BP support of 99.8%, while *Actinodaphne forrestii* and *Lindera megaphylla* were in the same lineage with *Litsea glutinosa* and *Litsea tomentosa* with BP support of 64.8%. *Litsea cubeba* nested within two other species of *Lindera* (*Lindera reflexa* and *Lindera fructicosa*) that had been analyzed by Li *et al.* (2004), with BP support of 64.8%. The other species of *Litsea*, including two species analyzed by Li *et al.* (2004), formed two monophyletic groups with BPs of 99.0% and 92.4%. Polytomies were also shown by several species of *Lindera.* The phylogenetic positions of other genera analyzed by Li *et al.* (2004) such as *Laurus*, *Iteadaphne* and *Umbellularia*, were obscure in the tree obtained.

MPR of phenetic character evolution in Litsea *and related genera*

Two phenetic characters (arrangement of flower parts and number of anther cells), which have been used to distinguish genera in tribe Laureae in most classifications of Lauracae, were applied for MPR analyses (Figs. 5-6). The evolution of these phenetic characters was examined by superimposing the characters on a combined *matK* and ITS tree based on our own data, since the combined data provided a better-resolved tree than either single data set. Trimerous flowers, which occur in *Actinodaphne*, *Lindera* and *Litsea*, were shown to be more primitive than the dimerous flowers of *Neolitsea* (Fig. 5). In number of anther cells, four-celled anthers, which are distributed in the tree genera, *Actinodaphne*, *Litsea* and *Neolitsea*, were estimated to be more primitive than the two-celled anthers of *Lindera* (Fig. 6).

Discussion

Molecular trees based on matK *and ITS data*

ITS data had a greater number of parsimony-informative sites (17.6%) than *matK* data (2.9%). The ITS region, however, was more homoplasious, as indicated by lower CI and RI values (Table 4). The *matK* tree demonstrated more highly supported clades, but the relationships among some lineages were not well resolved. The ITS tree showed better-resolved relationships but with lower bootstrap support. The combined *matK* and ITS showed a better-resolved tree than was generated by a single data set. The combined tree showed clear positions for each section of *Litsea*,

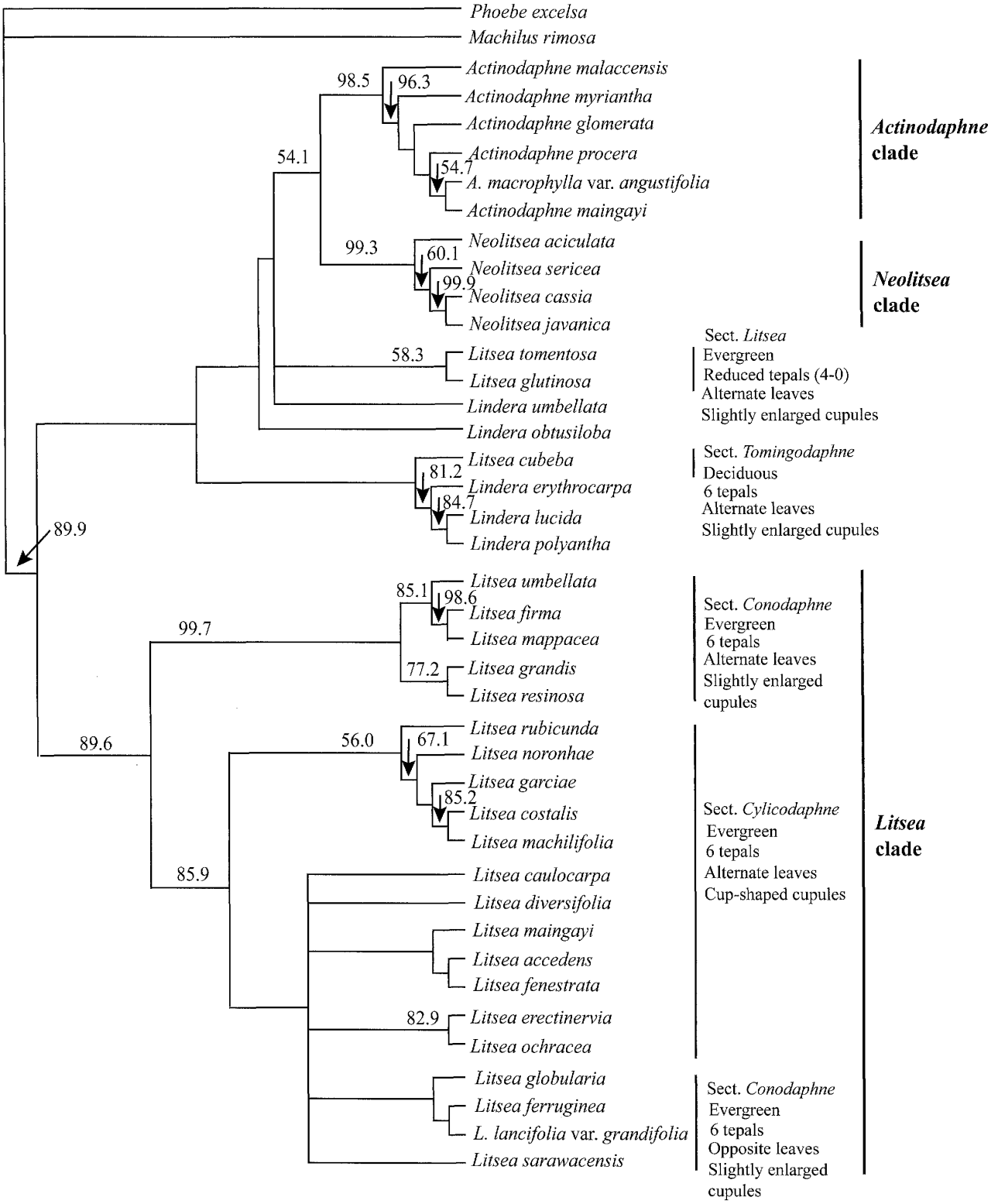

FIG. 3. Strict consensus tree (cladogram) of 12 trees derived from combined analysis of *matK* and ITS sequences (length = 620; CI = 0.548; RI = 0.699). Internal support was examined by bootstrap analysis of 1,000 replicates. Bootstrap values are shown above branches. Vertical bars to right circumscribe sections in *Litsea* and main clades.

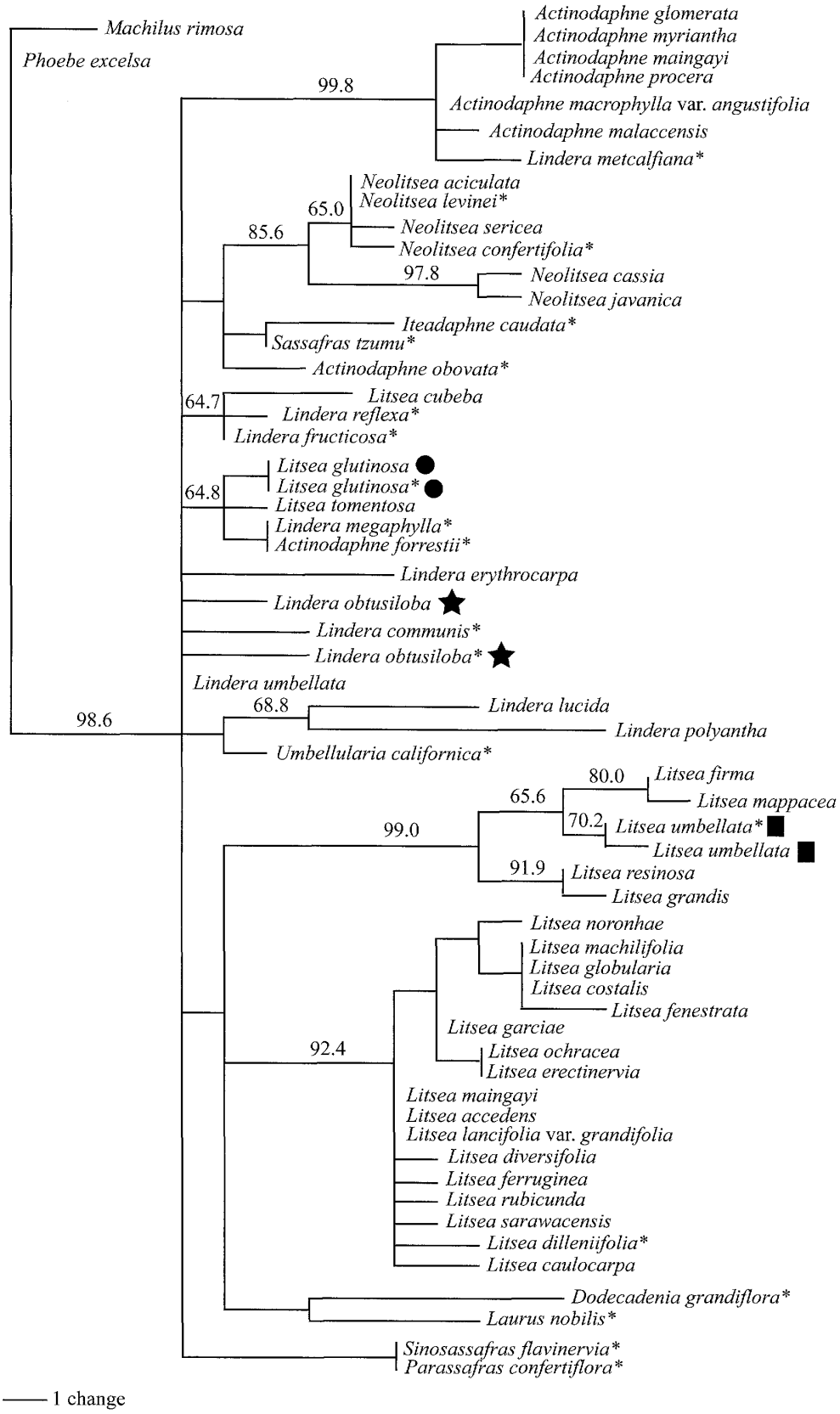

FIG. 4. One of 180 most parsimonious trees derived from analysis of *matK* sequences including species analyzed by Li *et al.* (2004), which are indicated by asterisks (length = 125; CI = 0.912; RI = 0.932). Internal support was examined by bootstrap analysis of 1,000 replicates. Bootstrap values are shown above branches. Branch length corresponds to number of nucleotide substitutions; scale bar is shown on lower left. Black shapes indicate species analyzed by us and by Li *et al.* (2004).

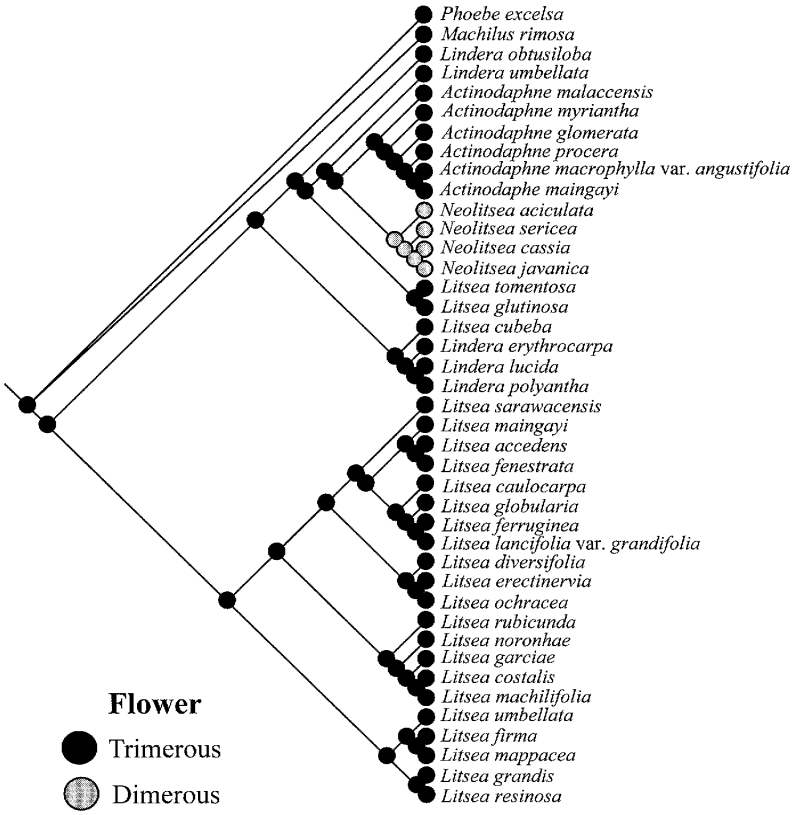

FIG. 5. Distribution of arrangement of flower parts plotted on one of the most parsimonious trees based on our combined *matK* and ITS data. Character status of each taxon is indicated by small circles in lower left.

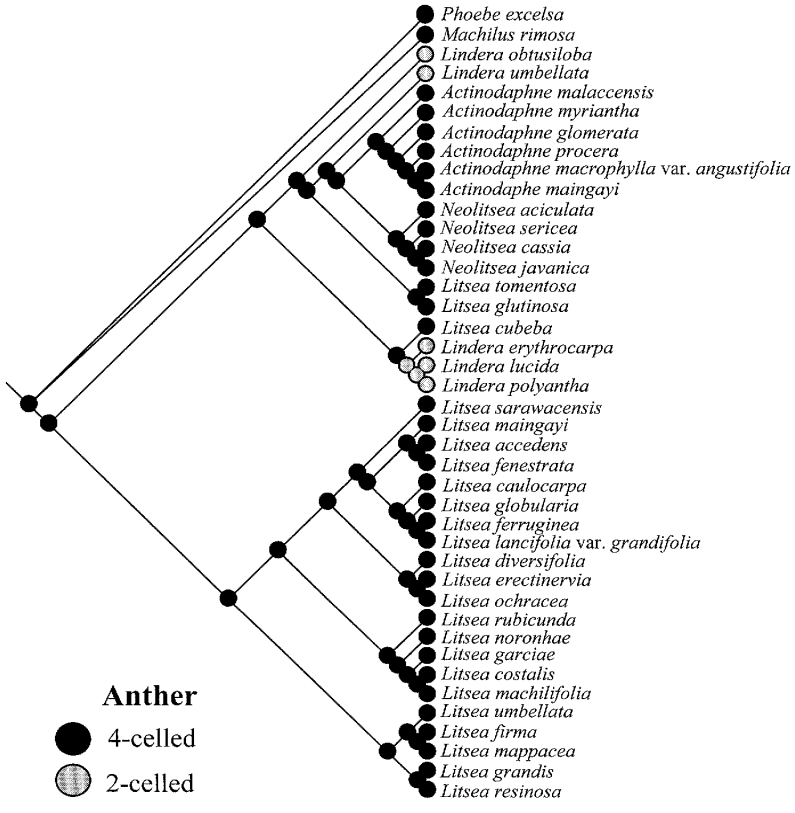

FIG. 6. Distribution of anther characters plotted on one of the most parsimonious trees based on our combined *matK* and ITS data. Character status of each taxon is indicated by small circles in lower left.

as well as relationships of *Litsea* to other genera. The molecular tree obtained by the combined analysis of *matK* and ITS data is therefore used for the remainder of the discussion.

In former classifications of Lauraceae, e.g., Bentham (1880), Pax (1891), Kostermans (1957) and Hutchinson (1964), great importance was given to the number of anther cells in delimiting genera. Recently, Li (1985, 1995), Tsui (1987) and Hyland (1989) also used this character. Rohwer *et al.* (1991), Li & Li (1991) and van der Werff & Richter (1996), however, argued that the number of anther cells is not an important character, since infrageneric variation in the number of anther cells per anther has been observed in several genera of Lauraceae (i.e. *Caryodaphnosis*, *Cinnamomum, Persea* and *Urbanodendron*). Our results supported the latter opinion.

From our results, some species of *Litsea* were found to be closely related to several species of *Lindera.* In Li & Christophel (2000), genera with two-celled and four-celled anthers were intermingled in phylogenetic trees based on morphological characters. Similarly, Li *et al.* (2004) reported molecular trees that showed intermingling of these characters.

The status of sections of Litsea *in the Malesian region and their relationships to* Lindera

According to Li *et al.* (1982), sect. *Conodaphne* was treated as having members with alternate or opposite leaves and non- to slightly enlarge fruiting cupules. In our combined molecular tree, the separation of sect. *Conodaphne* was strongly supported. Species with having leaves of sect. *Conodaphne* formed a clade with high BP support. Species with opposite leaves of sect. *Conodaphne* were joined with members of sect. *Cylicodaphne* with having alternate leaves and cup shaped fruiting cupules, with moderate BP support. Besides disagreeing with the classification of Li *et al.* (1982), in which opposite-leaved species were included in sect. *Conodaphne*, our results also indicated that the shape of the cupule is not a useful character for distinguishing these two sections of *Litsea* as defined in previous classifications, since this character did not reflect phylogenetic relationships among these sections.

Litsea sections, *Tomongodaphne* and *Litsea*, were also found to be closely related to some species of *Lindera* (Fig. 3). The close relationship of *Litsea* to *Lindera* has been suggested by many taxonomists as mentioned herein. Section *Tomingodaphne*, represented by *Litsea cubeba*, is distinguished from other sections of *Litsea* by being deciduous leaves. *Litsea cubeba* was shown to be closely related to *Lindera polyantha*, *Lindera lucida* and *Lindera erytrocarpa*, but the BP support was less than 50%. Members of sect. *Litsea,* represented here by *Litsea glutinosa* and *Litsea tomentosa*, and distinguished from other sections by having reduced number of tepals showed monophyly in the *matK* and combined *matK*-ITS trees with low BP support. Although *Litsea glutinosa* and *Litsea tomentosa*, were found to be closely related to some species of *Lindera*, the relationships among them are still vague. This unresolved *Lindera* clade might be due to our limited number of samples of *Lindera.* The sections of *Litsea* and their relationship to *Lindera* should therefore be reexamined. It is also necessary to find additional synapomorphies for a new classification.

Monophyly of the genera Actinodaphne *and* Neolitsea

Based on the *matK* and ITS data, Li *et al.* (2004) reported both *Actinodaphne* and *Neolitsea* to be polyphyletic. Li *et al.* (2006), based on the ITS and the external transcribed spacer (ETS) regions of ribosomal DNA of the nuclear genome data, also showed that *Actinodaphne* is polyphyletic. In contrast, Li *et al.* (2007), also based on ITS and ETS data, found that *Neolitsea* to be monophyletic. Recognition of *Actinodaphne* and *Neolitsea* as monophyletic genera was strongly supported by all our molecular trees (*matK*, ITS, and combined *matK* and ITS trees). Conflicts were thus observed between our results and those of Li *et al.* (2004) and Li *et al.* (2006). We assume that contamination by fungal ITS in their dataset might explain the conflicts, as we discuss in detail below. Our combined *matK* and ITS data also suggest that the two genera, *Actinodaphne* and

Neolitsea are sisters, but with low BP support.

Most taxonomists (e.g. Rohwer 1993, van der Werff 2001) have considered *Neolitsea* to be more closely related to *Litsea* than to *Actinodaphne*. *Actinodaphne* was described as having perulate buds, whorled leaves, trimerous flowers, four-celled anthers and disc- to cup-shaped cupules. *Neolitsea* is distinguished from other genera by having perulate buds, clustered leaves, dimerous flowers, four-celled anthers and disc-shaped cupules. Species of *Litsea* usually have non-perulate buds, alternate or opposite leaves, trimerous flowers, four-celled anthers and flat to deeply cup-shaped cupules. Our results support the classifications of Kostermans (1957), Hutchinson (1964) and Li *et al.* (1982), which distinguish *Neolitsea* as different from *Litsea*.

Comparisons with the Chinese Litsea *complex*

The analysis of the *matK* data from our dataset and that of Li *et al.* (2004) demonstrated molecular trees with a different topology from the one obtained only from our data. It was odd and difficult to explain why the Chinese *Lindera metcalfiana* was nested among the Malesian members of the *Actinodaphne* clade with very high BP support of 99.8%. Morphologically, *L. metcalfiana* is different from species of *Actinodaphne*. Besides having two-celled anthers, *L. metcalfiana* has alternate leaves and non-perulate buds as compared to the four-celled anthers, verticillate leaves and perulate buds of *Actinodaphne*. In contrast, members of *Actinodaphne* from China did not nest among the members of the same genus that we analyzed from the Malesian region. *Actinodaphne forrestii* and *Lindera megaphylla*, both species examined by Li *et al.* (2004), joined with the Malesian species *Litsea* of sect. *Litsea*. Moreover, both the *matK* and ITS sequences of *A. forrestii* and *L. megaphylla* were similar. Since these two species have been placed in different genera and are morphologically distinct, sharing almost identical sequences, especially in the rapidly evolving ITS region (Baldwin *et al.* 1995), seems unlikely. Based on homology searching at DDBJ (http://www.ddbj.nig.ac.jp) we also found that the ITS sequence data of *Neolitsea confertifolia*, *Lindera tienchuanensis* and *Sinosassafras flavinervia* reported by Li *et al.* (2004) were more similar to some species of fungi (*Aspergillus* spp.) than to species in the *Litsea* complex. Li *et al.* (2004) had apparently amplified and sequenced the ITS regions of fungi, which contaminated some of their plant samples. We regarded their ITS data to be unreliable, and therefore excluded them from our combined analysis. Further molecular examination of the Chinese species of *Litsea* complex is needed.

Neolitsea confertifolia and *Neolitsea levinei*, which were analyzed by Li *et al.* (2004), nested within *Neolitsea* (three species from Malesia and one species from Japan) in our study with high bootstrap supporting the monophyly of *Neolitsea*, even after including the Chinese species. *Litsea umbellata* (sect. *Conodaphne*) and *Litsea dillenifolia* (sect. *Cylicodaphne*) from China, which were analyzed by Li *et al.* (2004), also nested within the same sections of *Litsea* from the Malesian region with high BP support. The results indicate that members of sect. *Conodaphne* and sect. *Cylicodaphne* in Malesia and China are closely related.

Morphological character evolution in Litsea *and possibly related genera*

Li (1995) hypothesized parallel evolution at the generic and sectional levels between and within *Litsea* and *Lindera,* the two main genera of tribe Laureae. He also considered that all other genera of tribe Laureae developed from these two large genera, which may be the reason for the difficulty in delimiting genera using traditional morphological data. Reconstruction of the evolution of flower parts arrangement in *Litsea* and related genera suggests that trimerous flowers are more primitive than dimerous flowers. Two genera in tribe Laureae have dimerous flowers, *Neolitsea* and *Laurus*. Li (1985) and Li (1995) considered that *Neolitsea* and *Laurus* to be, respectively, derived from species of *Litsea* and *Lindera* with trimerous flowers. Our MPR of phenetic character evolution supported the derivation of dimerous flowers in *Neolitsea* from trimeous flowers.

It was also shown by the MPR analysis that

four-celled anthers are more primitive than two-celled anthers. In our plant study, genera with four-celled anthers were *Actinodaphne*, *Litsea* and *Neolitsea*; two-celled anthers were observed only in *Lindera*. Li (1985) proposed that *Litsea* and *Lindera*, the main genera in tribe Laureae, developed from *Parasassafras* and *Sinosassafras*, respectively. The former genus has four-celled anthers and the latter genus has two-celled anthers. These genera were considered to share *Sassafras* as common ancestor. *Sassafras* has two- or four-celled anthers. Li (1985) therefore considered lineages with two-celled anthers and those with four-celled anthers developed separately in the course of evolution of this plant group. Li (1985) did not indicate more the primitive type of anther. Rohwer (1993), however, considered the two-celled anther to have originated from four-celled anthers by reduction of the upper pollen sacs, as observed in *Persea*, by reduction of the lower pollen sacs, as observed in *Urbanodendron*, or by lateral fusion of adjacent pollen sacs, as observed in *Brassiodendron* and *Ediandra*. Our results showed that two-celled anthers were recently derived from four-celled anthers, and thus support the hypothesis of Rohwer (1993).

In conclusion, our results do not support the previous classifications of *Litsea* and related genera. Polyphyly of the sections was clearly shown except for sections *Litsea* and *Tomingodaphne*. Polyphyly of the genera *Litsea* and *Lindera* was also suggested. Major revision of both *Litsea* and *Lindera* is needed, based on increased sampling and additional molecular and morphological data. In our study, serious problems in using the ITS region for phylogenetic analyses were also documented. The universality of the ITS primers may sometimes facilitate PCR amplification of contaminants (Mort & Crawford 2004). Careful handling of samples and checking sequence data to insure they are free from contaminations is necessary when using ITS.

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